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14. ABSTRACT
During the last research year, we have made progress in two main areas. First, we have shown that neu-specific T cells can significantly inhibit growth of spontaneous mammary tumors; we plan to conduct T cell infusion studies in the spontaneous tumor model whenever possible. Second, we have made advances in understanding how and why the *ex vivo* cytokine environment in which the T cells are cultured impacts their clinical efficacy. We had hypothesized that the *ex vivo* cytokine milieu in which antigen-specific T cells are expanded in can greatly influence their function and clinical activity *in vivo*. We found that by simply adding IL-21 to IL-2 in culture, or by depleting IL-10 in the culture, we induced significant changes to the cytokine profile of those cultured T cells, which in turn enhanced their antitumor efficacy. IL-21 may enhance efficacy via induction of two proinflammatory cytokines, IL-17 and TNF-a. Anti-IL-10 antibody may enhance the clinical efficacy of the T cells via the inhibition of IL-10 production. For all 9 T cell culture conditions tested, there was uniformly high induction of IL-5, IL-6, IL-10, IL-13, IFN-g, TNF-a and GM-CSF, and low induction of IL-7, IL-12, IL-15 and IL-2. Overall, we discovered that the choice of culture cytokines in which T cells are grown for adoptive transfer studies is critical.

15. SUBJECT TERMS
spontaneous tumor, neu-transgenic mice, neu antigen, CD4+ T cells, cytokines, T cell culture, IL-21, anti-IL-10, T cell therapy, T cell infusion.

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Introduction

The purpose of this study is to evaluate mechanisms of generating $CD4^+$ neu-specific T cells *ex vivo* and to evaluate their anti-tumor efficacy *in vivo* in the neu-transgenic (neu-Tg) mouse model. Despite advances made in breast cancer research, relapse of micrometastatic breast disease remains to be a major hurdle. In animal models, adoptive T cell therapy has been shown to be an effective treatment for breast cancer. However, the potency of antitumor specific T cell cells can be limited by the number and type of T cells generated *ex vivo*, by endogenous T regulatory cells (Tregs) in the tumor microenvironment dampening the immune response, and the supoptimal ability of infused T cells to home to tumor and mediate tissue destruction. In addressing some of these challenges, the study has three specific aims: (1) to generate neu-specific CD4+ Th1 T cells in neu-Tg mice and determine whether infusion of these cells results in a long lasting anti-tumor response and epitope spreading; (2) to determine whether depletion of Tregs prior to infusion of tumor-specific CD4+ Th1 cells will enhance *in vivo* T cell proliferation and persistence thus augmenting anti-tumor therapeutic efficacy; and (3) to determine whether neu-specific T cells can home to micrometastatic tumor *in vivo* and elicit tumor cell apoptosis. The key accomplishments for the year 2 research period are detailed below.

Body

Specific Aim 1: To generate neu specific CD4+ Th1 T cells in neu-Tg mice and determine whether infusion of these cells results in an anti-tumor response and epitope spreading.

A.1. To elicit a high magnitude neu specific Th1 response after active immunization in preparation for *ex vivo* expansion.

A.2. To modify the culture environment to augment the proliferation of Th1 CD4+ neu specific T cells.

A.3. To determine whether infusion of neu specific Th1 T cells results in an anti-tumor response and epitope spreading.

Much of Subaims A.1 and A.3 were conducted and detailed in the Year 1 progress report. For A.1, what remains is the elucidation of the optimal vaccine adjuvant(s). This will be a major focus in Year 3 (see Future Directions). For A.3, in addition of the progress we have made in the evaluation of neu-specific T cells as therapy for implanted mammary tumors, we have now conducted studies to evaluate their antitumor efficacy in spontaneous mammary tumors. Previously for A.3, all the T cell infusion studies were assessed in the subcutaneous (s.c.) implant tumor model, which while is a suitable model, is not the most biologically relevant. This year, we have been able to evaluate the efficacy of infused neu-specific helper T (Th) cells on spontaneously arising tumors which are more similar, in histology and progression, to human breast cancers. A single dose of neu-specific T cells was significantly ($p<0.001$) effective at inhibiting tumor growth compared to naïve T cells (**Figure 1**). For future T cell infusion studies, we would like to evaluate spontaneous tumors, whenever possible, as they are more biologically relevant. However, we appreciate that it may be challenging to acquire sufficient tumors for study since the time period for spontaneous tumors to arise takes several months (approximately 6 months), as opposed to weeks (for implanted tumors). We will continue to use both models.

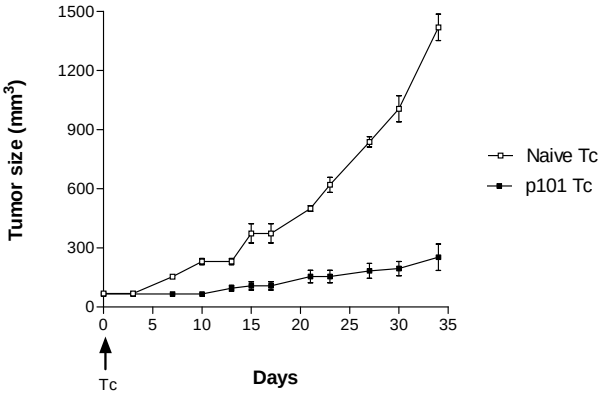
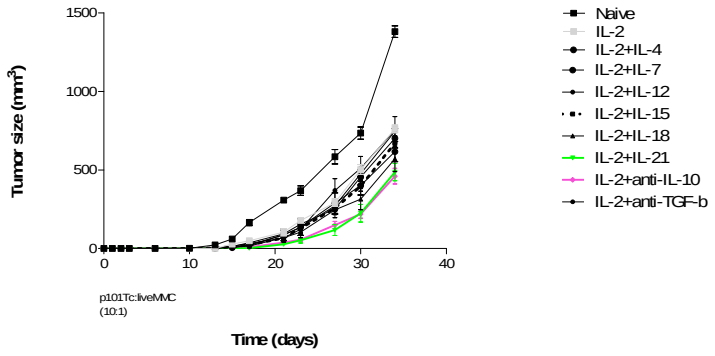


Figure 1. Neu-specific T cells inhibit growth of spontaneous mammary tumors. Mice (3 per group) bearing spontaneous tumors were injected i.v. with 2 x 10^7 p101 T cells (closed squares) or naïve (control) T cells (open squares). Tumor growth was followed. Each data point represents the mean (±SEM).

In the Year 2 research period, most of the significant progress has been made in Subaim A.2. This past year, we have begun to better understand how the *ex vivo* environment in which the T cells are cultured can impact their *in vitro* cytokine profile and growth potential, as well as their antitumor function *in vivo*. Prior to this, it had been unclear as to which cytokines were best suited during *ex vivo* T cell culture to promote the generation of maximally functional, high avidity, tumor antigen-specific T cells. Thus, we evaluated several cytokines, based on their unique properties to stimulate T cell differentiation, proliferation and/or function (see **Appendix, Table 1**). The cytokines and combinations evaluated included: 1) IL-2 alone; 2) IL-2+IL-4; 3) IL-2+IL-7; 4) IL-2+IL-12; 5) IL-2+IL-15; 6) IL-2+IL-18; 7) IL-2+IL-21; 8) IL-2+anti-IL-10; and 9) IL-2+anti-TGF-b. Anti-IL-10 and anti-TGF-b antibodies were included to deplete IL-10 and TGF-b that may have been secreted during T cell culture, thus counteracting their immunosuppressive properties. Culture supernatants were collected at various timepoints during the culture and set aside for subsequent evaluation by ELISA (Luminex).

A.



B.

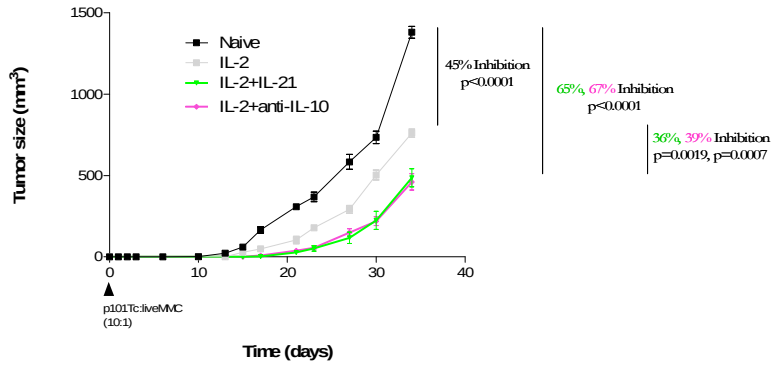


Figure 2. IL-2/IL-21 and IL-21/anti-IL-10 cultured T cells potently inhibit local tumor growth. A. Live MMC cells (1 x 10^6) were admixed with various *ex vivo* culture T cells (1 x 10^7) and implanted in neu-Tg mice (n=5 per group) s.c. on day 0. Tumor growth was followed. Each data point represents the mean (±SEM). **B.** Statistical comparison of IL-2/IL-21 and IL-2/anti-IL-10 groups to IL-2 alone and naive (non cytokine) control.

At the end of culture, *in vivo* T cell activity was assessed by admixing the various cytokine-cultured T cells with live tumor cells (10:1 T cell to tumor), implanting the cells s.c. in neu-Tg mice, and evaluating the extent at which the admixed T cells inhibited local tumor growth. **Figure 2A** shows that at day 34 following implant, mice which received naïve (non-cytokine stimulated) T cells had a mean tumor volume of 1380 mm^3. However, the groups which received the various cytokine-stimulated T cells had much smaller tumors, ranging from 352 – 760 mm^3. Compared to naïve T cells, IL-2 cultured T cells significantly inhibited tumor growth, by 45% ($p<0.0001$) (**Figure 2B**). However, the T cells which most effectively inhibited tumor growth were IL-2/IL-21 and IL-2/anti-IL-10 cultured cells; they suppressed tumor growth by 65% and 67%, respectively, compared to naïve cells; $p<0.0001$. These two cultures were the only ones which showed a significantly greater tumor inhibition over IL-2 culture ($p=0.0019$ and $p=0.0007$, respectively) (**Figure 2B**).

To assess if the clinical efficacy of the IL-2/IL-21 and IL-2/anti-IL-10 T cells might be due to a distinct cytokine profile we assessed, by Luminex, the type and magnitude of Th cytokine secretion. The supernatants evaluated were taken at the end of the *ex vivo* culture, prior to infusion. Among the 13 cytokines assessed were Th1 cytokines (IL-2, IL-12, IFN-g, GM-CSF), Th2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF), as well as others (e.g. IL-17). The heat map of the cytokine production (**Figure 3)** reveals some striking similarities among the cultures. There was a high induction of IL-5, IL-6, IL-10, IL-13, IFN-g, TNF-a, and GM-CSF, but very low (negligible) levels of IL-7, IL-12, and IL-15. IL-2 production was also uniformly low.

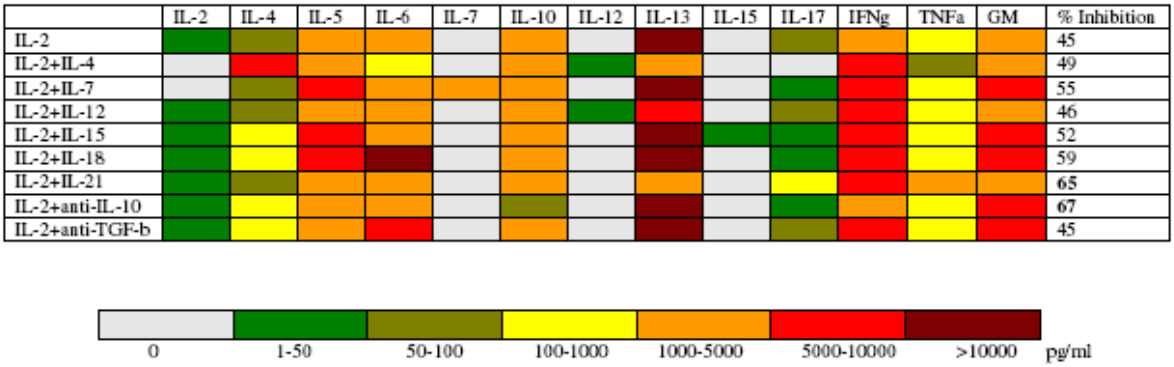


Figure 3. Multiplex cytokine analysis of cultured T cells. T cells were isolated and generated from spleens of p101 peptide-primed neu-Tg mice, and cultured in the various conditions (left column). Supernatants of cultured T cells were collected at the end of expansion (prior to *in vivo* infusion) for evaluation of cytokine secretion using Luminex technology. Shown is a heat map illustrating the magnitude of secreted cytokines detected (top row). The corresponding percentage of tumor inhibition for each cultured group is shown (right column).

Of note, while IL-17 production by the majority of the culture groups was low, the IL-21 culture was unique in that of the cytokines it induced the highest IL-17 production (**Figure 4A**) as well as highest TNF-a production (**Figure 4B**). Both of these are pro-inflammatory cytokines which play a key role in autoimmunity. The mechanism and relevance of IL-17 induction in the antitumor response are currently being investigated. We postulate that the unique secretion of these proinflammatory cytokines (IL-17 and TNF-a) by IL-2/IL-21 cultured T cells enhances their clinical efficacy *in vivo* (Figure 2), by eliciting and augmenting a proinflammatory microenvironment at the tumor site.

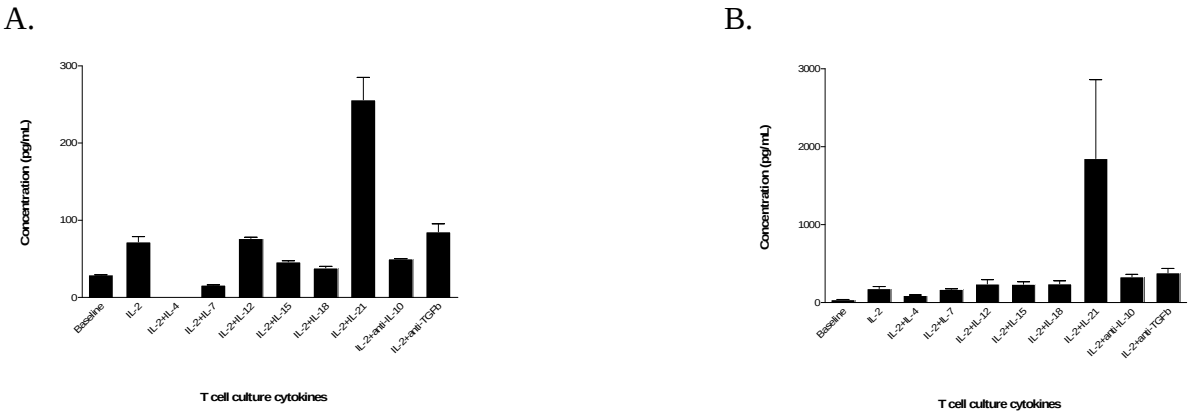


Figure 4. IL-2/IL-21 induce highly active antitumor T cells secreting autoimmune cytokines. T cells were isolated and generated from spleens of p101 peptide-primed neu-Tg mice, and cultured in the various conditions (x axis). Supernatants of cultured T cells were collected at the end of expansion (prior to *in vivo* infusion) to evaluate for cytokine secretion using Luminex technology. Shown are the level of IL-17 (**A**) and TNF-a (**B**) production by the cultured T cells.

The IL-2/anti-IL-10 cultured T cells were also unique from the rest in that IL-10 production was significantly downregulated (**Figure 5**). It is possible that blocking IL-10 production from the T cells rendered them more clinically effective *in vivo* (Figure 2).

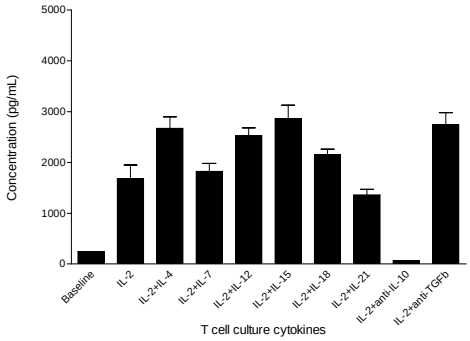


Figure 5. IL-2/anti-IL-10 induce highly active antitumor T cells that are inhibited in IL-10 secretion. T cells were isolated and generated from spleens of p101 peptide-primed neu-Tg mice, and cultured in the various conditions (x axis). Supernatants of cultured T cells were collected at the end of expansion (prior to *in vivo* infusion) to evaluate for cytokine secretion using Luminex technology. Shown is the level of IL-10 production by the cultured T cells.

Specific Aim 2: To determine whether depletion of Tregs prior to infusion of tumor specific CD4+ Th1 cells will enhance *in vivo* T cell proliferation and persistence, thus augmenting anti-tumor therapeutic efficacy.

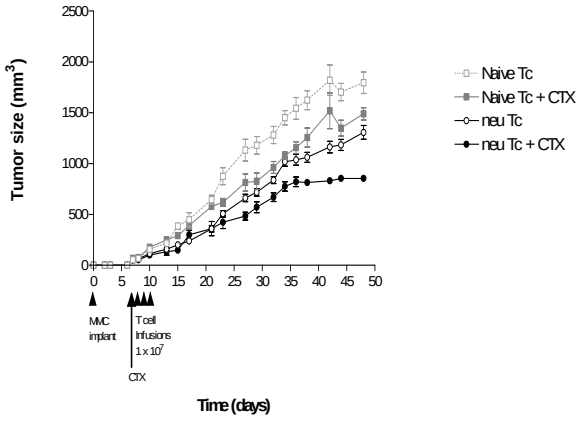


Figure 6. Inhibition of mammary tumors by neu-specific T cells in the presence of cytoxan pretreatment. Mice (5 per group) were given live MMC cells s.c. on day 0. On day 7 (arrow) following tumor implantation, some mice were injected with CTX (180 mg/kg dose) prior to infusion with 1 x 10^7 naïve (squares) or neu-specific T cells (circles) on days 8, 9, and 10. Tumor growth was followed. Each data point represents the mean (±SEM).

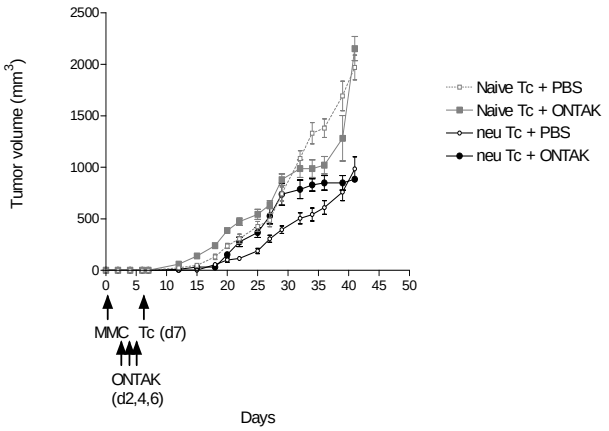


Figure 7. Inhibition of mammary tumors by neu-specific T cells in the presence of ONTAK pretreatment. Mice (5 per group) were given live MMC cells s.c. on day 0. On days 2, 4, and 6 (arrows) following tumor implantation, some mice were injected with ONTAK (5 μg dose) prior to infusion with 1 x 10^7 naïve (squares) or neu-specific T cells (circles) on day 7. Tumor growth was followed. Each data point represents the mean (±SEM).

Lymphodepletion with cytoxan prior to T cell infusion may create a homeostatic space to enable enhanced proliferation of infused T cells, and may augment antitumor efficacy of infused T cells. Thus, our goal was to evaluate if the efficacy of infused antigen-specific T cells could be augmented by cytoxan (CTX) pretreatment prior to T cell infusion. It did not appear that CTX pretreatment enhanced T cell efficacy (**Figure 6**). Nor did it appear that ONTAK pretreatment enhanced efficacy (**Figure 7**). However, these studies are preliminary and need to be repeated before any strong conclusions can be made. Also, since ONTAK is relatively expensive and its availability for clinical use is becoming rarer, in our future studies we plan to evaluate only CTX and anti-CD25 antibody as Treg-depleting agents.

Specific Aim 3: To determine whether neu-specific T cells can home to micro-metastatic tumor *in vivo* and elicit tumor cell apoptosis.

We have been unable to make much progress on this aim, due to the unavailability of the mouse PET scanner. There have been electronic malfunctions that have prevented its use for the past year. A new machine is being acquired by the Department of Nuclear Medicine which should become available for collaborative use in late November/December. In the meantime, in preparation, we have performed radioactive uptake studies (cut-and-count method) to evaluate the biodistribution of 3H-deoxyglucose and 14C-thymidine radiotracers in neu-Tg mice (feasibility test). The uptake of 3H-deoxyglucose was quantified in the tumors of mice following various therapies (doxorubicin therapy, neu-specific monoclonal antibody therapy, or PBS as control). **Table 2** (Appendix) shows the uptake expressed as injected dose per gram of tumor relative to injected dose per gram in blood.

Key Research Accomplishments

- A single infusion of neu-specific T cells can significantly inhibit growth of spontaneous mammary tumors
- Addition of IL-21 or anti-IL-10 antibody to IL-2 during T cell culture significantly enhances the clinical efficacy of those T cells
- IL-21 may enhance the clinical efficacy of the T cells via the promotion of IL-17 and TNF-a production
- Anti-IL-10 antibody may enhance the clinical efficacy of the T cells via the inhibition of IL-10 production
- Among the various cultured T cell groups that were tested, there was uniformly high induction of IL-5, IL-6, IL-10, IL-13, IFN-g, TNF-a and GM-CSF, and low production of IL-7, IL-12, IL-15 and IL-2.
- The cut-and-count method of radiotracer uptake/quantification was tested for 3H-deoxyglucose and was shown to be a feasible assay, with relative ease of use.

Reportable Outcomes

Publications:

Phan, V., Disis, M.L., and Lu, H. Immune Recognition of Breast Cancer. In: The Breast: Comprehensive Management of Benign and Malignant Diseases, In press.

Phan, V., Gad, E., Saddoughi, S., DeLong, J., Waisman, Z., and Disis, M.L. CD4+ Th1 HER2-specific T cells as a novel treatment for HER2-overexpressing breast cancer. Poster presentation at the *Era of Hope 2008 Meeting*, June 25-28, Baltimore, MD.

Phan, V., Gad, E., Davis, K., Delong, J., Gwin, W., Wallace, D., and Disis, M.L. Enhancing the clinical activity of tumor antigen specific T cells. Oral presentation at the *7th Biennial Ovarian Cancer Research Symposium*, September 4-5, 2008, Seattle, WA.

Conclusions and Future Directions

During the last funding year, we have made significant progress in discovering how and why the *ex vivo* cytokine environment in which the T cells are cultured impacts their clinical efficacy. We had hypothesized that the *ex vivo* cytokine milieu in which antigen-specific T cells are expanded in can greatly influence their function and clinical activity *in vivo*. This has turned out to be true. Simply by adding IL-21 to IL-2 in culture, or by depleting IL-10 in the culture, we induced significant changes to the cytokine profile of those cultured T cells, which in turn greatly impacted the clinical activity of those cells. Thus, the choice of culture cytokines in which T cells are grown for adoptive transfer studies is critical. Future directions include evaluating the antitumor efficacy of the IL-2/IL-21 and IL-2/anti-IL-10 cultured T cells on established, spontaneous tumors, as well as deciphering the effector T cell subgroup (e.g. is there expansion of Th17 cells in addition to Th1 cells?). Future directions also include evaluating the cytokine profile of the serum of T cell treated mice to assess whether it is similar to that of the *in vitro* cytokine profile. Efforts in elucidating the optimal vaccine adjuvants have not yet been completed (Aim A.1) and will be a major focus of next year's research. We plan to assess/compare vaccine adjuvants with different properties (e.g. depot effect, induction of dendritic cell (DC) maturation, induction of DC chemotaxis, induction of DC1 phenotype). Finally, major efforts will be focused on Aim 3, once a mouse imaging scanner becomes available again.

Appendices:

Table 1.

Cytokines	Properties
IL-2	Critical T cell growth factor; promotes function of effector CD8+ T cells
IL-4	Stimulates differentiation of naïve CD4 T cells into Th2 cells
IL-7	Regulates homeostatic proliferation of memory T cells
IL-12	Stimulates the production of IFN-g and TNF-a from T cells and NK cells
IL-15	Promotes proliferation and effector function of CD8+ T cells, NK, and NKT cells
IL-18	Induces IFN-g and accelerated memory CD8+ T cell proliferation
IL-21	Elicits concurrent humoral and cellular responses; promotes differentiation of naïve CD8 T cells into effector T cells

Table 2.

Tumors	% ID/g tumor: % ID/g blood
PBS mouse #1	15.6
PBS mouse #2	29.6
PBS mouse #3	12.7
7.16.4 mouse #1	8.2
7.16.4 mouse #2	30.9
7.16.4 mouse #3	10.9
Doxorubicin mouse #1	17.2
Doxorubicin mouse #2	6.4
Doxorubicin mouse #3	22.8